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(54) Title of the Invention: Base Sequences of DNA or Partially Denatured DNA

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Specification

1. Title of the Invention: Base Sequences of DNA or Partially Denatured DNA

2. Patent Claims

-1- A method of determining the bases of DNA or partially denatured DNA with the following characteristics. DNA or partially denatured DNA is marked radioactively and then subjected to base-specific cleaving, producing a cleaved section that has a radioactive marker, that is subsequently expanded on a supporting medium. Visible images of such separated and expanded rows are produced on radioactive film. The separated and expanded positions of the base-specific, cleaved sections that appear in such visible images are used to determine the base sequences of DNA or partially denatured DNA.

In such methods, the base-specific cleaved sections with the radioactive marker are separated and expanded on a supporting medium. At that point, the DNA or partially denatured DNA is cleaved specifically at each of its four types of bases that are its constituent elements. A mixture of the resulting cleaved sections is then separated and expanded simultaneously and in parallel on the same supporting medium. The separated and expanded row of said mixture is then taken as a standard of all of the separation and expansion positions. That row is then compared to the separation and expansion positions of the other base-specific cleaved sections that appear in the other separation and expansion rows, allowing for identification.

-2- A method of determining the base sequences of the DNA or partially denatured DNA described in Claim 1 of the Range of Patent Claims with the following features. The process in which the base-specific cleaved sections with radioactive markers or mixtures of base-specific cleaved sections are separated and expanded on a support medium is carried out using electrophoresis on a gel made of a macromolecular substance.

-3- A method of determining the base sequences of the DNA or partially denatured DNA described in either of Claims 1 or 2 in the Range of Patent Claims having the following features. The following were used in mixtures of base-specific cleaved sections for separating and expanding standard rows.

1) Guanine (G) specific cleaved sections, adenine (A) specific cleaved sections, thymine (T) specific cleaved sections as well as (C) specific cleaved sections.

The following were used as base-specific cleaved sections having four groups of radioactive markers.

2) Guanine (G) base-specific cleaved sections,
3) Guanine (G) specific cleaved sections + adenine (A) specific cleaved sections,
4) Thymine (T) specific cleaved sections + cytosine (C) specific cleaved sections,
5) Cytosine (C) specific cleaved sections.

-4- A method for determining the base sequences of the DNA or partially denatured DNA described in Claims 1 or 2 of the Range of Patent Claims with the following features. Mixtures containing the following base-specific cleaved sections are used to obtain standard separated and expanded rows. Base-specific cleaved sections having 4 groups of radioactive markers made up of the following are used in this method.

1) Guanine (G) base-specific cleaved sections, adenine (A) base-specific cleaved sections, thymine (T) base-specific cleaved sections as well as (C) base-specific cleaved sections.

The following was used as a base-specific cleavage having a radioactive marker.

2) Guanine (G) specific cleaved sections.
3) Adenine (A) specific cleaved sections.
4) Thymine (T) specific cleaved sections.
5) Cytosine (C) specific cleaved sections.

Base-specific cleaved sections having 4 groups of radioactive markers made up of the above are used in this method.

3. Detailed Description of the Invention

This invention pertains to methods of determining the base sequences of DNA or partially denatured DNA. More specifically, this invention pertains to methods of determining the base sequences of DNA or partially denatured DNA using autoradiography that employs radioactive film.

Autoradiography is already known as one method of forming a distribution row in at least a 1-dimensional direction on the support medium in order to obtain position information on the radioactive marked substance.

For example, radioactive markers are placed in macromolecular substances that come from an organism such as a protein or ribonucleic acid and those radioactive marked macromolecular substances, their inducers or their cleavages are subjected to gel electrophoresis or other separation process in which they are separated and expanded on a gel support medium. That gel support medium and highly sensitive X-ray film are layered for a specific period of time, which exposes said film. Methods have been developed and are in actual use that separate and identify the macromolecular substance or determine the characteristics or molecular weight of the macromolecular substance based on the radioactive marker position information on the gel support medium that is obtained from the position of the exposure.

In recent years particularly, autoradiography has been used effectively to determine the base sequences of DNA or other ribonucleic acid (or partially denatured DNA from them, (hereafter described as "DNA or other ribonucleic acid").

Known methods of determining the base sequences of DNA by using this sort of autoradiography include the Maxam-Gilbert Method and the Sanger-Coulson Method.

In these methods, the DNA, which has a double helix structure made up of two strands of molecules, and the two strands of molecules are made up of structural units that have bases made up of four types of bases. These are adenine (A), guanine (G), cytosine (C) and thymine (T). The space between these two strands of molecules is bridged by a hydrogen bond between these 4 types of bases. Additionally, these methods determine the base sequences by making use of a structural characteristic of DNA in which the hydrogen bonds between each of the structural units are only manifested in two types of combinations: G – C and A – T.

For example, the Maxam-Gilbert Method is implemented using the methods described below.

First, a base containing a radioactive isotope of phosphorous (P) is bonded to the end of one side of a strand of molecules of the DNA or partially denatured DNA for which the base sequence row is to be determined. This makes it possible to produce radioactively marked substances. Next, a specific chemical process is performed on these radioactively marked substances so that they are cleaved specifically at the bonds between each of the structural units of the chain molecules. For example, the following four types of base-specific cleaved sections are

obtained.

1) Guanine (G) specific cleaved sections
2) Guanine (G) specific cleaved sections + adenine (A) specific cleaved sections
3) Thymine (T) specific cleaved sections + Cytosine (C) specific cleaved sections.
4) Cytosine (C) specific cleaved sections.

Next, the above base-specific cleaved sections are separated and expanded in parallel on the same support medium using gel electrophoresis. Each of the base-specific cleaved sections separate and expand in one-dimensional direction, forming separated and expanded rows. (However, these cannot be seen.) Next, the aforementioned method is used, and an autoradiograph is produced on X-ray film or other radioactive film so that they can be seen.

In the row/series described above, the autoradiograph produced is capable of showing the 4 types of separated and expanded rows as visible images of the following.

1) Separated and expanded rows showing the expansion position of the guanine (G) specific cleaved sections.
2) Separated and expanded rows showing the expansion position of the guanine (G) specific cleaved sections and the adenine (A) specific cleaved sections.
3) Separated and expanded rows showing the expansion position of the thymine (T) specific cleaved sections and the cytosine (C) specific cleaved sections.
4) Separated and expanded rows showing the expansion position of the cytosine (C) specific cleaved sections.

Next, the separated and expanded rows of numbers 1) and 2) are compared, which allows the identification of the expanded position of the guanine-specific cleaved sections and the adenine-specific cleaved sections. Then, the same sort of comparison is made with the separated and expanded rows numbers 3) and 4), which identifies the expanded positions of the thymine specific cleaved sections and the cytosine specific cleaved sections.

That is, each of the expanded positions of the guanine (G) specific cleaved sections, the adenine (A) specific cleaved sections, the thymine (T) and the cytosine (C) specific cleaved sections is identified. Then the phoretic distance of each of the cleavage products is determined based on the molecular weight. Based on that knowledge, the sequences of all of the bases in fixed positional relationships are determined using the terminal points of the chain molecules to which the radioactive isotopes is bonded. This allows the identification of the all of the base arrays of the object in question.

However, using the above radioactive photographic method in the autoradiography to determine the base sequences of the DNA, offers the significant advantage of allowing the visual measurement of the position information using the molecular units of the radioactively marked substances.

Be that as it may, however, it is easy for bubbles to be generated inside the gel when a gel made of macromolecular substances is used as the separation and expansion support medium. Additionally, this gel has no capacity to support itself, so normally, the separation and expansion is performed with it sandwiched in between two sheets of a glass or other support. However, it frequently happens that distortion in those supporting bodies causes a lack of uniformity in the gel, which means that the radioactive marking substances will not necessarily be separated and expanded in a uniform fashion across the support medium. For reasons of this sort, for example, the distance traveled by the separated and expanded rows at both ends of the support medium will be shorter, relative to that distance traveled by the separated and expanded rows near the center. This "smiling" effect is a frequent occurrence. Additionally, there are times when the current applied to the entire support medium is not uniform during separation and expansion using electrophoresis. In such cases, the separation and expansion conditions will vary locally over the support medium, which leads to distortions in the separated and expanded rows produced.

For these reasons, it is not always a simple matter to identify the expanded positions of the base-specific cleaved sections based on a comparison of each of the aforementioned sorts of separated and expanded rows. This is why the used of the above identification process has been considered a significant problem in conventional autoradiography methods of determining the base sequences of DNA or partially denatured DNA.

As described above, this invention provides a method for determining the base sequences while eliminating the problems in the procedures of determining the base sequences of DNA or of partially denatured DNA.

The method of identifying base sequences of DNA or partially denatured DNA of this invention has the following characteristics. DNA or partially denatured DNA is marked with a radioactive marker and then subjected to base-specific cleaving, producing cleaved sections that have radioactive markers. These sections are separated and expanded on a support medium and the separated and expanded rows are turned into visible images on radioactive film. The base sequences of the DNA or partially denatured DNA are determined based on the separated and expanded positions of the base-specific cleaved sections that appear in the visible images.

In this method, the base-specific cleaved sections having radioactive markers are separated and expanded on a support medium. During that separation and expansion, the DNA or partially denatured DNA is cleaved specifically for each of four types of bases that make up their constituent units. A mixture of these cleaved sections is separated and expanded simultaneously and in parallel on the same support medium. The separation and expansion positions that appear in the separated and expanded rows of said mixture are used as a reference to make a comparative identification of the separation and expansion positions of the base-specific cleaved sections that appear in the other separated and expanded rows.

The following is a detailed description of this invention.

The DNA or partially denatured DNA that serves as the object of the base sequence identification method of this invention is the same sort of method used in conventional methods of identifying the base sequences of DNA or partially denatured DNA used in conventional autoradiography and it is not particularly restricted. In other words, it includes all types of DNA and the partially denatured DNA that is made by cleaving those DNA types at specific locations. Additionally, it is already public knowledge that there is a process of supplying those types of DNA and partially denatured DNA with radioactive markers and that the resulting radioactively marked substances can be cleaved at base-specific sites to produce base-specific cleaved sections that have radioactive markers.

By way of explanation, a simple description of the above process is disclosed in the following citation.

"Analysis of DNA Base Sequences: Reading and Expressing Genetic Information in the Original Language," by Kin'ichiro MIURA, Gendai Kagaku (Modern Chemistry), September 1977, pp. 46 – 54 (Published by Tokyo Chemical Associates Group, Co., Ltd.)

Additionally, detailed descriptions of the above process can also be found in the following citation.

"Methods in Enzymology, Vol. 65, Part I (Academic Press, New York, London, Toronto, Sydney, San Francisco, 1980).

Furthermore, examples of methods for separating and expanding the above radioactively marked substances using a support medium are also described in the aforementioned citations, but typical methods include for example, gel support media (either layered or columnar), acetate or other polymer body, electrophoresis using filter paper or any other support media or thin layer chromatography using silica gel support media, as well. Of these, the preferred methods are electrophoresis methods in which the support media are formed using macromolecular gel.

Below, we will look at examples of methods of determining the base sequences of DNA using the Maxam-Gilbert Method described above as a method for determining the base sequences of the DNA or partially denatured DNA of this invention. We used the following 5 types of cleaved sections (or mixtures of cleaved sections) as combinations of typical base-specific cleaved sections for determining those base sequences. Our descriptions focus on these.

1) guanine (G) specific cleaved section
   + adenine (A) specific cleaved section
   + thymine (T) specific cleaved section
   + cytosine (C) specific cleaved section.
2) guanine (G) specific cleaved section.
3) guanine (G) specific cleaved section + adenine (A) specific cleaved section.
4) thymine (T) specific cleaved section
   + cytosine (C) specific cleaved section.
5) cytosine (C) specific cleaved section.

By way of explanation, the mixture in 1) above contains 4 types of base-specific cleaved sections and is used as a standard in the formation of separation and expansion rows.

First, the DNA to be used in our study was marked with a ^{32}P radioactive marker. For this, we used normal chemical means and, denaturing specifically at each of the four types of bases that are the constituent elements of DNA, we produced the above base-specific cleaved sections 2) – 5) above (each with a radioactive marker).

Next, these base-specific cleaved sections were mixed in a suitable manner, producing the mixture in 1) above.

Subsequently, the mixture in 1) and the base-specific cleaved sections in 2) – 5) above were separated and expanded in parallel using electrophoresis on the same gel support medium. This yielded separated and expanded rows (however, these could not be seen) of the mixture and the base-specific cleaved sections above.

Next, we exposed X-ray film to the support medium that had been used to form these separated and expanded rows. The exposure was performed at low temperature (for example -70 ~ -90° C) over a period of several days. An autoradiograph (visible image) was then made on the X-ray film after it had been developed.

Figure 1 shows an autoradiograph that shows the 5 separated and expanded rows (electrophoresis rows) that formed with the base-specific cleaved sections of the radioactively marked DNA and mixtures of them.

That is to say, in Figure 1, each of the rows, from the first row to the fifth row in order, are shown as follow.

(1) – (G) specific cleaved sections.
(2) – (G) specific cleaved sections
+ (A) specific cleaved sections.
(3) – (G) specific cleaved sections
+ (A) specific cleaved sections
+ (T) specific cleaved sections
+ (C) specific cleaved sections.
(4) – (T) specific cleaved sections
+ (C) specific cleaved sections.
(5) – (C) specific cleaved sections.

Of the above separation and expansion rows, the row 3 is the one that is a separation and expansion of a mixture containing all of the base-specific cleaved sections (G, A, T and C). It will be used as an internal standard row (reference row) in determining the base sequences of these separated and expanded rows.

Below we offer an example of the identification method using the internal standard row described above to identify the separated and expanded samples that appear as bands in each of the separated and expanded rows.

First, we will compare the internal standard row 3 with the developed and expanded row 2, which is next to it. All of the base-specific cleaved sections are contained in the internal standard row, so all of the expanded positions of the separated and expanded sample, the (G) specific cleaved sections and the (A) specific cleaved sections, that appear in row 2 should correspond to some of the separated and expanded samples appearing in the internal standard row. For this reason, misalignment between row 2 and the internal standard row can be easily corrected by finding the correspondence between the rows in the separated and expanded sample in row 2.

Next, we compare the internal standard row 3 with the separated and expanded row 4 next to it. All of the base-specific cleaved sections are contained in the internal standard row, so all of the expanded positions of the separated and expanded sample, the (G) specific cleaved sections and the (A) specific cleaved sections, that appear in row 4 should correspond to some of the separated and expanded samples appearing in the internal standard row. Furthermore, the fact that row 2 and row 4 are mutually exclusive can be used to draw an even more accurate correspondence between the entire separated and expanded row 4 and the separated and expanded internal standard row. In this way, misalignment between row 4 and the internal standard row can be easily corrected by drawing a correspondence between the entire separated and expanded sample in row 4 and the separated and expanded sample in the internal standard row.

Next, by comparing the separated and expanded rows in row 2 with those in row 1, it will be possible to identify the expanded positions of the (G) specific cleaved sections and the (A) specific cleaved sections. Additionally, by making the same sort of comparison between the separated and expanded rows in row 4 with those in row 5, it will be possible to identify the expanded positions of the (TG) specific cleaved sections and the (C) specific cleaved sections. Note also that by referring appropriately to the degree of misalignment between the separated and experimented rows determined as described above, it will be extremely easy to compare and identify these samples during this process of identifying the base-specific cleaved sections and to improve the precision.

In other words, the method of identifying base sequences of DNA and partially denatured DNA of this invention described above allows the following. The process of identifying base-specific cleaved sections of DNA

or partially denatured DNA is extremely easy and the precision is improved remarkably compared with procedures used in methods based on the separation and expansion of base-specific cleaved sections of the 4 groups (four types) made up of G, A, T and C or methods based on the separation and expansion of the base-specific cleaved sections of the 4 conventional groups made up of G, G + A, T + C and T.

Furthermore, in the description and example above, we described an example in which the separated and expanded row of the mixture that served as the internal standard row had been placed in the center, but there is no particular need to place the internal standard row in the center. It could be placed in any position for other separated and expanded rows.

It is also possible to place several internal standard rows. For example, internal standard rows could be alternated with the normal separation expansion rows. This would make the process of identifying the base-specific cleaved sections of the DNA or partially denatured DNA using this invention even easier and the precision would be improved remarkably as well.

Note that in the method for identifying the base sequences of the DNA or partially denatured DNA of this invention and the method of identifying DNA base sequences using the combinations described above (G + A + T + C, G, A + G, T + C and C) are just one example of the methods for determining DNA base sequences. The method of this invention, for determining base sequences is not limited to the above combinations and a variety of combinations are possible. It is also possible to determine the base sequences in the same way using methods that conform to the above method using such combinations.

In other words, such combinations of base-specific cleaved sections that have radioactive markers, for example might involve using combinations of the following 4 groups of cleaved sections.

Guanine (G) specific cleaved sections,
Adenine (A) specific cleaved sections,
Thymine (T) specific cleaved sections, and
Cytosine (C) specific cleaved sections.

Additionally, in the example above, we used 5 rows of radioactively marked substance to form the separation and expansion rows that were separating and expanding in a one-dimensional direction on the supporting medium. However, the separation and expansion rows are not restricted to these 5 rows. There could be more than 5 rows or less than 5 rows.

That is, for example, it would be possible to determine the position of just the guanine (G) in the DNA or partially denatured DNA using the following combinations.

(a) (G) specific cleaved sections, or
(b) (G) specific cleaved sections,
+ (A) specific cleaved sections
+ (T) specific cleaved sections
+ (C) specific cleaved sections.

In other words, the phrase determination of the base sequences of the DNA or partially denatured DNA of this invention, refers to not only to the determination of the base sequences of all bases, but also includes the determination of position of just a portion of the bases as shown above.

Note also that our descriptions thus far have used a single support medium in our examples, showing the determination of the base sequences of a single type of DNA or partially denatured DNA, but it would also be possible to determine the base sequences of 2 or more types of DNA or partially denatured DNA simultaneously using a single supporting medium.

Additionally, the analysis of the autoradiograph produced as a visible image on radioactive film can be performed visually with the human eye, but it is also permissible to use a method of determination that employs a scanning densitometer or similar device.

4. Brief Description of the Figures

Figure 1 is a schematic diagram of an autoradiograph showing the separation and expansion rows (electrophoresis rows) that were formed when the base-specific cleaved sections of DNA and mixtures of them, which had been radioactively marked, were undergoing separation and expansion on the support medium.

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Cleaner Copy of Drawing (Content Unchanged)

Figure 1

[See original for figure]

[right side of page]

Procedural Correction Form

January 25th, 1983

To: Patent Office Official: Kazuo WAKASUGI

1. Disclosure of Incident

58-1335
Patent Application (15) Submitted January 8th, 1983.

2. Title of the Invention

Method of Determining Base Sequences of DNA or Partially Denatured DNA

3. Party Making Corrections

Relationship to Patent: Patent Applicant
Minoru Onishi, Representative
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4. Representative

Yasuo YANAGIKAWA, Patent Attorney (7467) [Yasuo Yanagikawa stamp]
2 – 14 Mitsuya Yotsuya Bldg. 8 Fl. (358) 1798/9

5. Date Correction Ordered: Voluntary

6. Increase in Number of Inventions due to Correction: None

7. Objects to be Corrected: Drawing

[Patent Office Stamp (partial)]

8. Content of Corrections: Submitted official drawing.